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(54) Title: HAPTEN/ANTI-HAPTEN AFFINITY LINKING IN CELL SEPARATION

(57) Abstract

The invention relates to a method of positively isolating a target cell type from a mixed population of cells wherein, sequentially or simultaneously, a hapten is bound to either an insoluble support or said target cell and an anti-hapten is bound to said hapten and to the other said support and said target cell, whereby said support and said target cell are isolated from the mixed population of cells and the target cell is released from said support by the addition of hapten or hapten analogue. This method is particularly useful in the isolation of infectious agents, malignant cells or protective cell populations.

Hapten/antihapten affinity linking in cell separation.

This invention relates to a method of linking target substances in a manner which can be reversed with minimal destructive effect, and in particular to a method of linking a target cell to a support, in a manner which permits a desired cell-type to be positively selected.

In biochemistry, as in other fields, it is frequently desirable to link two chemical entities, for example in isolation or purification or in immobilisation of substances on solid supports. In particular, it is often required to isolate cells by attaching them to substances assisting in their isolation and to isolate the cells subsequently in viable form. However, the substances so attached to the cells tend to hinder cell reproduction and viability.

Such linkage has often been accomplished using affinity binding, that is by means of a pair of binding partners which are separately attached to the substances to be linked and bind when brought into contact. Such binding partners include, typically, biotin and avidin or streptavidin. This system has the advantage that biotin is a small molecule which can readily be covalently bound to proteins and other substances of interest and the binding constant of such a system is very high so that binding is particularly efficient. However, this has the corollary that the binding is virtually irreversible without very significant destruction of the target substances. Antigen-antibody binding partners are also of use in such linkage but, again, it is difficult to reverse the linkage, e.g. by change of pH, without destructive effects. There is thus a requirement for a method of binding two substances, typically proteins, in a manner which can

readily be reversed without significant destruction of the bound substances.

It has now been found that hapten/anti-hapten binding pairs provide a particularly appropriate reversible linkage system in that reaction of substances linked by such a system with an excess of the hapten, a small molecule providing efficient and rapid reaction kinetics, or with an analogue of the hapten having a greater affinity for the anti-hapten, readily breaks the linkage under mild conditions avoiding destruction of proteins or other sensitive species present. In particular, where one of the bound substances is a molecule on a cell surface, the cell can be bound, for example to a reporter substance or a solid support and subsequently liberated with its reproductive potential undiminished.

It is known to use particles to isolate cells, but the methods used heretofore can only generally be applied in a negative isolation procedure. In other words, unwanted cells can be removed from a cell preparation by incubating the cells with antibodies specific for these unwanted cells and thereafter removed by means of the particles. The unreacted cell population will be left behind more or less purified. Attempts have been made to use particles to positively isolate cells by employing antibodies against the cells to be isolated. To liberate the cells from the particles, the cell/particle rosettes have needed to be incubated over night at 37°C to lead to separation of the cells from the particles. In some cases the cells detach from the particles, but in many cases the cells do not.

We have now found that the hapten/anti-hapten linkage system enables cells in a positive selection procedure to be easily liberated from the particles or other solid support.

According to a first aspect of the invention there

is therefore provided a method of positively isolating a target cell type from a mixed population of cells wherein, sequentially or simultaneously, a hapten is bound to either an insoluble support or said target cell and an anti-hapten is bound to said hapten and to the other of said support and said target cell, whereby said support and said target cell are reversibly linked, the support and bound target cell are isolated from the mixed population of cells and the target cell is released from said support by the addition of hapten or hapten analogue.

The term "cell" is intended to encompass both prokaryotic and eukaryotic cells and viruses. However, it should be noted that the method according to the invention may be used to isolate sub-cellular components such as mitochondria and nuclei, and macromolecules, such as proteins and nucleic acids.

It should be noted that the anti-hapten may be a complete anti-hapten antibody or a hapten-binding fragment, e.g. an $F(ab)_2$ or Fv fragment, thereof.

The insoluble support or the target cell may be covalently bound to the hapten by a preliminary chemical reaction. Thus, for example, the hapten 4-hydroxy-3-nitro-phenylacetic acid (NP) can be activated, e.g. by forming an activated ester such as NP-caproyl-O-succimidyl ester (NP-CAP-O-Su) and reacted with an insoluble support or target substance cell carrying free NH_2 groups.

The anti-hapten may then, in one embodiment of the method, be reacted with the hapten carrying support to provide a reagent capable of binding to target cells via other binding interactions. Thus, for example, where the anti-hapten is a complete antibody with its Fc region intact, such a reagent will react with cells carrying Fc receptors and thereby bind the cells to the support. Alternatively, the anti-hapten may be bound to the target cell initially and then reacted with the

hapten-carrying support.

On the other hand, the anti-hapten may be attached to the insoluble support, advantageously via the Fc region of the intact antibody, leaving the hapten-binding portions free. In this case, the support may carry anti-Fc antibody, i.e. antibody produced in an animal of a species different from that producing the anti-hapten antibody. Thus, if the anti-hapten antibody is a mouse antibody or mouse-human chimeric antibody, the support may carry sheep-antimouse antibody to bind the anti-hapten antibody to the support while the hapten may be bound to the target cell directly or indirectly via free NH_2 or other appropriate functional groups.

It will be appreciated that the insoluble support and target cell, normally may be reacted simultaneously with the anti-hapten antibody instead of in separate stages.

The target cell, once bound to the support may be released by a mild change in conditions caused by the addition of excess hapten or by the addition of a haptan analogue. Preferably the target cell/support complex is first washed to remove contaminants before it is released from the support by excess hapten or by addition of hapten analogue.

The anti-hapten antibody may be, for example, of polyclonal or monoclonal origin. Monoclonal antibodies are preferred because of their homogeneity.

The hapten may be linked directly, normally covalently, to the insoluble support or the target cell or it may be linked indirectly, for example by being covalently coupled to an antibody which binds to the insoluble support or the target cell. Likewise, the anti-hapten may be attached directly to the insoluble support or target cell or it too may be linked indirectly, for example by being coupled to an antibody which binds to the insoluble support or the cell.

Indirect linkage of the hapten or anti-hapten to

the target cell by means of a cell-specific antibody or fragment thereof is a particularly preferred embodiment of the invention, since the use of specific anti-cell antibodies or antibody fragments enables a desired cell population to be positively selected from a mixed population of cells. In order to leave the smallest possible residue on the cell after liberation, it is preferred that the hapten or anti-hapten is linked to the cell by the smallest effective anti-cell antibody fragment, e.g. an $F(ab)_2$ or Fv fragment.

In this specification the term "hapten" is intended to encompass any small molecule which by itself cannot stimulate antibody synthesis but will combine with an antibody formed by immunising an animal with an antigenic conjugate of the hapten and some other substance e.g. a protein such as keyhole limpet haemocyanin. Hapten analogues are analogues of such molecules which will also combine with the anti-hapten antibody. Ideally, when the method is used to isolate living cells, the hapten (and, if used, its analogue) is non-toxic.

Examples of well known haptens include aminobenzene sulphonates and corresponding arsenates and carbocylates (Landsteiner, J.Exp.Med. 1936 63: 325) and molecules containing nitrophenyl and dinitrophenyl groups such as nitrophenylacetic acid and dinitrophenyl acetic acid, in particular 4-hydroxy-3-nitrophenyl acetic acid. (J. Klein, Immunology, "The science of self-non-self" John Wiley & Son 1982). Derivatives of these compounds preferably have linker groups to enable attachment of hapten to the antigenic protein used to produce the anti-hapten e.g. capryl-OH groups. Analogues of such haptens which have higher binding affinities to anti-hapten include 5-iodo derivatives of such phenolic haptens e.g. 4-hydroxy-5-iodo-3-nitro-phenylacetic acid or its cap-OH derivative. It is, of course, desirable that the hapten and hapten analogue should be water-

soluble since, in general, the coupling and uncoupling reactions will be effected in aqueous media.

The affinity of the hapten or a hapten analogue for the binding partner can be tailored so that the excess hapten or hapten analogue added to the mixture containing immobilised target cell will have a greater affinity for the binding partner than the hapten or hapten analogue used to link the cell to the insoluble support. Even without this sort of tailoring of affinity, an excess of hapten or hapten analogue pushes the equilibrium of bound and free cell towards a higher proportion of free target cell. The fact that haptens are small molecules allows an unbound excess of hapten or hapten analogue to readily compete with the hapten or analogue attached to the target cell or insoluble support and effect displacement.

The insoluble support may be a surface on a plate or tube such as a microtitre well although more preferably it is provided by a particulate material. The particulate material may be, for example, beads of agarose gel or finely divided apatite. It is preferred that the particles are monodisperse and, advantageously superparamagnetic; Dynabeads M-450 being an example of such beads. The manufacture of monodisperse superparamagnetic beads is described in EP 83901406.5 (Sintef).

A second aspect of the invention therefore provides an insoluble support, preferably particles and more particularly magnetic particles, coated with hapten.

The invention also provides, in a third aspect thereof, an insoluble support, preferably particles and more particularly magnetic particles, coated with anti-hapten.

A fourth aspect of the invention provides an insoluble support, preferably particles and more particularly magnetic particles, coated with anti-hapten bound to hapten which is bonded to anti-cell antibodies

or fragments thereof.

The invention provides, in a fifth aspect thereof a kit comprising; (i) an insoluble support according to the second aspect of the invention, (ii) anti-hapten capable of being bound to a target cell, and (iii) an effective amount of hapten or hapten analogue.

A sixth aspect of the invention provides a kit comprising; (i) an insoluble support according to the third aspect of the invention, (ii) hapten capable of being bound to a target cell, and (iii) an effective amount of hapten or hapten analogue.

A seventh aspect of the invention provides a kit comprising; (i) an insoluble support according to the fourth aspect of the invention, and (ii) an effective amount of hapten or hapten analogue.

It will be clear to a person skilled in the art that "an effective amount of hapten or hapten analogue" will be that which will lead to the release of target cell from the insoluble support.

The method of the invention has many uses in the field of cell isolation. For example, the method may be used to isolate infectious agents such as bacteria or viruses in order to quantitate them or characterise their infectivity, toxicity or susceptibility to drug treatment. The method can also be used for isolation of malignant cells or cell populations specific for different diseases and to characterise these cells further without interference from other contaminating cells. Also, the method may be used to isolate protective cell populations from an individual or form a group of individuals; the isolated population can then be expanded and/or potentiated before being returned to the patient under treatment. Such protective cell populations can for example be monocytes/macrophages, lymphocytes or bone marrow stem cells. In the case of lymphocytes one can use the method to isolate antigen-specific cells which may have antitumor activity and use

said cells for cancer treatment. Infectious agents and malignant cells can also be isolated and studied for drug susceptibility in order to choose the most effective treatment strategy.

The invention will now be described by way of non-limiting examples in which the method is used to isolate cells, but as mentioned earlier, the method is equally applicable for the isolation of bacteria and viruses or other substances.

Below is described a rapid and generally applicable method for the positive isolation of cells by means of monodisperse superparamagnetic particles.

The method of isolation has two main steps, one in which particles and the desired cell are linked or complexed to each other by hapten/antihapten antibodies optionally covalently bonded to anti-cell antibodies or fragments thereof such as $F(ab)_2$ or Fv fragments, and another where the isolated cell/particle rosettes are gently disrupted by incubation for a short time with a competing hapten or hapten analogue solution.

Noncomplexed cells are separated from the corresponding complexed ones by use of a magnet when the particles are magnetized or by other physio-chemical means when they are not. The isolated completed cells are stripped from the particles by incubation with excess of hapten which will break the hapten/antibody bonds present in the cell/particle complexes. The particles are then separated from the free cells by the same method used to isolate the complexes of cell particles i.e. magnet or other physio-chemical means.

The affinity between anti-hapten antibody and hapten can be tailored by suitable selection systems or by empirical trials. Such antibodies can also cross react with chemically related hapten analogues which have either lower or higher affinity towards the antibody compared to the hapten used for immunization (in which case the antibodies are "heteroclitic"). The

hapten-binding antibodies should preferably be of hybridoma origin, but polyclonal antibodies may also be used. For optimal separation, the hapten used in linking the cells and particles should have lower affinity for the hapten-antibody than the hapten used to competitively liberate the cells from the cell/particle complex.

The following Examples are given by way of illustration only.

Example 1

This example describes the isolation of cells which each have Fc receptors on their membranes. Such Fc receptors can react with the Fc part of immunoglobulins of different species. This specificity is taken into account when the anti-hapten antibody is chosen. Hapten is coupled to particles and then reacted with anti-hapten antibody. The Fc-part of this antibody will then react with the Fc-receptor on the cells to be isolated and the cell/particle complex can be disrupted by incubation with a solution of free hapten.

The isolation was performed by coupling hapten (NP) to particles having free NH_2 groups. Activated haptens such as NP-Cap-O-Su (4-hydroxy-3-nitro-phenacetyl-caproyl-O-succimidyl ester, e.g. supplied by Cambridge Research Biochemicals Cat. No. PA18040) OR nip-Cap-OSu (4-hydroxy-5-iodo-3-nitro-phenacetyl-caproyl-O-succimidylester, Cat. No. PA18140 were used to attach the NP groups to the particles. Paramagnetic hapten coated particles were reacted with chimeric antibodies, i.e. antibodies which are constructed using a mouse antigen binding part (V-region) connected to an human effector (C-region) part. These chimeric anti-hapten antibody bearing particles were then incubated with Fc-receptor positive cells from humans whereby Fc-receptor positive cells became linked to the particles and could then be isolated by means of a magnet leaving the Fc-receptor negative cells behind. The Fc-receptor

positive cells were then "stripped" from the particles by incubation with excess of hapten (NIP, NP, NIP-Cap-OH or NP-Cap-OH) and the particles separated from the cells by a magnet. The presence of excess hapten leads to the separation of the cells from particles in a gentle way with little or no loss in cell viability.

The anti-hapten antibodies can be chosen from other species to isolate Fc-receptor positive cells reacting with immunoglobulins from that species. Immunoglobulin class and subclass specificity of the Fc-receptor positive cells can also be selected for by employing anti-hapten antibodies of one class or subclass only.

Example 2

As a modification of Example 1, Dynabeads M-450 which can be coated by standard methods with, for example, BSA-NIP or BSA-NP were used. The rest of the procedure was as described in Example 1.

Example 3

Cells were separated by coupling anti-hapten antibodies either to secondary antibodies such as sheep anti-mouse antibodies or directly to monoclonal antibodies. The monoclonal antibody is specific for the type of cell to be isolated. The particles, which in this Examples were paramagnetic Dynobeads were haptenised as in Example 1. The particles were loaded with hapten/anti-hapten/sheep anti-mouse and the cell-preparation reacted with monoclonal antibodies which specifically reacted with the cell type to be isolated. Complexes (rosettes) formed between the cells to be isolated and the particles. As the particles were paramagnetic, the complexes were isolated by the use of a magnet. The cells were then separated from the particles by incubation with an excess of free hapten.

Example 4

Compared to Example 3 the coupling was turned round in this Example by coating the particles with anti-hapten antibodies and haptenising either the secondary

antibody (i.e. sheep anti-mouse) or the monoclonal anti-cell antibody. When the anti-hapten antibody and the anti-cell antibody are both mouse antibodies, the anti-hapten antibodies would preferentially be applied in the form of $F(ab')_2$ fragments prepared by standard pepsin digestion at pH 4.0-4.8, and the sheep anti-mouse antibody should be specific for the Fc-part of mouse IgG prepared by immunizing with Fc-fragments made by papain digestion or trypsin digestion of mouse IgG. Using this precaution, the sheep anti-mouse antibodies will only react with the cell/bacteria/virus specific antibodies and not with mouse $F(ab')_2$ anti-hapten fragments.

The particles were loaded with anti-hapten antibodies/haptenised secondary antibodies or with anti-hapten antibodies/haptenised secondary antibodies/monoclonal anti-cell antibodies. In the first case the cells were first reacted with the specific antibody and then reacted with the sensitized particles. In the second case the sensitized particles are reacted directly with the cells. After the polymer particle/cell complex has been separated from non complexed cells by the use of a magnet, the cells can be stripped off the particles by incubation with excess of free hapten. The method which leaves the lowest number of linking materials on the finally isolated cells will be most favourable for further biological use. In this example, the cells are relatively free of linking materials where the cell specific antibody is haptenised (for example in the NP/NIP-system with NP/NIP-Cap-O-Su supplied from C R B).

Claims:

1. A method of positively isolating a target cell type from a mixed population of cells wherein, sequentially or simultaneously, a hapten is bound to either an insoluble support or said target cell and an anti-hapten is bound to said hapten and to the other said support and said target cell, whereby said support and said target cell are isolated from the mixed population of cells and the target cell is released from said support by the addition of hapten or hapten analogue.

2. A method as claimed in claim 1 wherein said insoluble support is provided by particulate material.

3. A method as claimed in claim 3 wherein said particulate material is selected from agarose beads, finely divided apatite and magnetic particles.

4. A method as claimed in claim 3 wherein said particulate material is magnetic particles.

5. A method as claimed in any one of claims 1 to 4 wherein said hapten is selected from aminobenzene suphonates, arsenates or carbolylates or molecules obtaining nitrophenyl and dinitrophenyl groups.

6. A method as claimed in anyone of claims 1 to 5 wherein said hapten and/or said anti-hapten are linked indirectly to said insoluble support and/or said target cell.

7. A method as claimed in claim 6 wherein said indirect linkage is accomplished by means of an

antibody or fragment thereof which binds to the insoluble support or target cell.

8. A method as claimed in any one of claims 1 to 7 wherein hapten or anti-hapten is indirectly bound to said target cell by means of anti-cell antibodies, or fragments thereof, specific for the cell-type to be isolated.

9. A method as claimed in any one of claims 1 to 5 wherein said anti-hapten is bound by means of its Fc region to target cells carrying Fc receptors.

10. An insoluble support coated with hapten.

11. An insoluble support coated with anti-hapten.

12. An insoluble support coated with anti-hapten bound to hapten which is bonded to anti-cell antibodies or fragments thereof.

13. An insoluble support as claimed in any one of claims 10 to 12 being magnetic particles.

14. A kit comprising; (i) an insoluble support according to claim 10, (ii) anti-hapten capable of being bound to a target cell (iii) an effective amount of hapten or hapten analogue.

15. A kit comprising; (i) an insoluble support according to claim 11, (ii) hapten capable of being bound to a target cell, and (iii) an effective amount of hapten or hapten analogue.

16. A kit comprising; (i) an insoluble support according to claim 12 and (ii) an effective amount of hapten or hapten analogue.

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 90/01171

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 5/00, C 12 Q 1/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 N; C 12 Q, G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO, A1, 8602091 (IMMUNOTECH) 10 April 1986, see page 3, line 14 - line 35; claim 10	1-4,6-8, 11-13
Y		5,9,10, 14
X	WO, A1, 8704628 (FRED HUTCHINSON CANSER RESEARCH CENTER) 13 August 1987, see page 3, lines 11-17 and 25-36, pages 4, 6 and 26-29	1-4,6-16
Y		1-16
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* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
5th October 1990	20. 11. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Natalie Weinberg

International Application No. PCT/EP 90/01171

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
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X	DE, A1, 3705686 (BOEHRINGER MANNHEIM GMBH) 1 September 1988, see page 3, line 47 - line 50; claim 2	10,11
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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/EP 90/01171

SA 38381

Is annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
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